EXAMINING ALLELOPATHIC COMPETITON OF A RED TIDE ALGA WITHIN TWO DIFFERENT MARINE COMMUNITIES

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EXAMINING ALLELOPATHIC COMPATITON OF A RED TIDE ALGA WITHIN TWO DIFFERENT MARINE COMMUNITIES

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1. ABSTRACT

Blooms of the marine dinoflagellate *Karenia brevis* exist in two distinct habitats: far offshore where blooms initiate at low densities and inshore where dense blooms are driven by wind and water currents. Two competing hypotheses could explain variation seen in competitor species response to *K. brevis* allelopathy: offshore species are more susceptible to allelopathy because they have not evolved a mechanism to combat allelopathy, or inshore species are more susceptible to allelopathy because *K. brevis* evolved the allelopathic mechanisms to combat these species specifically. The allelopathic effects of *K. brevis* were observed on competitor species from each environment. Nine species, four offshore and five inshore, were exposed to *K. brevis,* but separated by mesh so that no cellular contact occurred between *K. brevis* and competitors. The growth of one inshore species and one offshore species was significantly inhibited by *K. brevis* allelopathy. There was no difference between inshore and offshore species response to allelopathy and therefore the hypotheses were rejected. However, treatments from both habitats responded similarly in that there fluorescence was unusually high, indicating *K. brevis* allelopathy causes sublethal damage to photosystem II.

2. INTRODUCTION

Karenia brevis is a single celled algal species responsible for nearly mono-specific "red tide" blooms in the Gulf of Mexico. Blooms occur yearly, most often in the fall (Tester and Steidinger 1997). *K. brevis* releases toxins called brevetoxins, a causative agent of fish kills and neurotoxic shellfish poisoning in humans and other mammals that consume affected fish, making these blooms harmful both to the environment and to human health (Landsberg 2002). However, while brevetoxins are detrimental to humans and higher order members of the marine community, other plankton species, like *Asterionellopsis glacialis,* are less affected by brevetoxins (Prince et al 2010).

Karenia brevis releases multiple chemical compounds to deter their competitors, a mechanism of competition called allelopathy (Kubanek et al 2005). Allelopathy is a chemical defense employed by plants and phytoplankton, including many marine species, such as the *Alexandrium* species of dinoflagellates (Muller 1969; Tillmann and John 2002). In harmful algal blooms, allelopathy is not likely the cause of bloom initiation, when population densities are low but could be an important factor in bloom maintenance at high cell concentrations (Jonsson at al 2009).

Allelopathic compounds vary between species, between individual strains of species, and are difficult to isolate in pure form, due to low concentrations exuded and decomposition in extracted fractions (Prince et al 2010). These allelochemicals are different from toxins produced by some phytoplankton, causing a range of effects, from reduced motility to cell lysis (Prince et al 2008). Allelopathic compounds isolated from *K. brevis* are small, polar, organic molecules but not brevetoxins (Prince et al 2010). However, the allelopathic effects of these compounds on competitors vary between

competitor species, years, and field collections. It has been established that the variation in allelopathic potency is not a product of variations in bloom intensity or concentration of brevetoxins (Prince et al 2008).

Karenia brevis blooms in two distinct habitats in the Gulf of Mexico. The blooms begin in the deep water offshore and move inland by wind and water currents to the shallower water. Salinities, nutrient levels, and light availability are different in each environment, as well as the members of the planktonic community. *Leptocylindrus danicus, Chaetoceros affinis, Stephanopixus turris, Rhizosolenia setigera* are diatom species isolated from the offshore bloom sites of *K. brevis* (NCMA isolation). *Asterionellopsis glacialis*, *Skeletonema grethae*, *Odontella aurita*, *Amphora* sp., and *Thallassiosira* are diatom species isolated from inshore *K. brevis* bloom sites (Badylak 2007). These environmental and community differences could affect allelopathic impacts. Much is known about *K. brevis* allelopathic effects on inshore species but very little is known about the impact these compounds have on species present offshore. Understanding variation in response to *K. brevis* allelopathy will provide insight into bloom dynamics and why *K. brevis* blooms when and where it does. This study proposes two competing hypotheses: (1) offshore phytoplankton species are could be more affected by *K. brevis* allelopathy than inshore species because they have not evolved a defense mechanism due to limited exposure to dense populations of *K. brevis*, or (2) inshore phytoplankton species are more susceptible to allelopathy from *K. brevis* because allelopathy evolved in *K. brevis* as a defense mechanism against inshore competitors. The goal of this study was to investigate a possible explanatory mechanism for variation in response to allelopathy from *K. brevis*.

3. METHODS

3.1 Organisms

Phytoplankton species were purchased from National Center for Marine Algae and maintained in Maine seawater (35 ppt salinity) supplemented with L1 nutrients (with silicates for diatom species). During the experiment, cultures were maintained in artificial seawater (35 ppt salinity). Inshore diatom species used were *Amphora* sp. strain CCMP 129, *Asterionellopsis glacialis* strain CCMP 137, *Odontella aurita* strain CCMP 1796, *Skeletonema grethae* strain CCMP 775, and *Thalassiosira* sp. strain CCMP 1055. Offshore diatom species used were *Chaetoceros affinis* strain CCMP 159, *Leptocylindrus danicus* strain CCMP 1856, *Rhizosolenia* cf. *setigera* strain CCMP 1694, and *Stephanopyxis turris* strain CCMP 815. *Karenia brevis* strain CCMP 2228 was used.

3.2 Experiment comparing response of inshore and offshore species to allelopathy

Competitors were exposed to live *K. brevis* separated by a mesh screen so that allelopathic compounds could pass through, but cells could not. A cage was constructed from a 50 ml polystyrene centrifuge tube. The closed, tapered end of the tube was sawed off and 5 μm nylon mesh was heat sealed to the sawed end. A small hole was drilled in the side (at the 30ml mark) to accommodate a long needle (600 μm inner diameter). Competitor species were cultured in 100 ml Pyrex bottles and the cage was suspended from the top of the bottle, such that the competitors were outside the cage. When the water level inside and outside the cage equilibrated, *K. brevis* was added to the cage. Approximately 20 ml of media was inside the cage at the start of the experiment.

Outside of the cage, bottles were inoculated with 9 ml competitor culture in 68 ml L1 media. Treatment cages were inoculated with 3 ml *K. brevis* and control cages were inoculated with 3 ml of 65% dilute L1 media. Total volume was 80ml. Initial biovolume of *K. brevis* and each competitor tested were within one order of magnitude. To monitor the growth in response to *K. brevis*, 3 ml of competitor species culture was sampled through a needle (600 micrometer inner diameter) every other day, over a period of 8 to 10 days. *R. setigera* was sampled by lifting the cage and sampling through a sterile pipette (to maintain cellular viability). Three ml of *K. brevis* culture was sampled on the first and last day of the experiment. *In vivo* fluorescence was measured, and samples were preserved in Lugol's dye and cells counted using an Olympus IX50 inverted microscope to determine cell density. A Turner Trilogy fluorometer was used to measure chlorophyll a fluorescence or raw fluorescence (i.e. uncalibrated fluorescence).

3.3 Analysis

Cell concentration data from microscopy counts were used to calculate percent growth (eqn, 1), exponential growth rate (eqn. 2, Miller 2004), and relative percent growth (eqn. 3, modified from Poulson et al 2010) and exponential growth (eqn. 4, modified from Poulson et al 2010) were calculated in order to assess the differing susceptibility among species. Unpaired two sample t-tests were used to assess *K. brevis* effects on individual species compared to dilute media control and to analyze inshore versus offshore effects, with the factors being relative percent growth and relative exponential growth rate. Fluorescence was analyzed using 2-way repeated measures ANOVA with Sidak's multiple comparison post hoc test and unpaied t-tests at individual time points.

Equation 1 $$\left(\frac{Final\_cell\_concentration - Initial\_cell\_concentration}{Initial\_cell\_concentration}\right) \times 100$$

Equation 2 $$(1/t) * (\ln(\frac{\text{Final\_cell\_concentration}}{\text{Initial\_cell\_concentration}}))$$

Equation 3 $$1 - (\frac{\%growth\_control - \%growth\_final}{\%growth\_control})$$

Equation 4

$$1 - (\frac{\exp onential\_growth\_rate\_control - \exp onential\_growth\_rate\_treatment}{\exp onential\_growth\_rate\_control})$$

4. RESULTS

3.1 Individual species effects

Growth of *A. glacialis* and *L. danicus* were significantly inhibited by the presence of *K. brevis* (Figure 1). The exponential growth rates of *A. glacialis* and *L. danicus* calculated from cell concentrations counted under a microscope were reduced in the presence of *K. brevis* by 24% and 39% respectively (t-test, n=6, p=0.021 and p=0.048, Figure 1). The percent growth of *A. glacialis* calculated from cell counts was inhibited by the presence of *K. brevis* by 20% (t-test, n=6, p= 0.011, Figure 1). *Skeletonema grethae*, *C. affinis*, and *S. turris* all trended toward inhibition (Figure 1). *Thalassiosira* sp., *Amphora sp*., *O. auritum*, and *R. setigera* were not noticeably affected by *K. brevis* (Figure 1). The fluorescence of *S. turris* and *Amphora* sp. were enhanced in the presence of *K. brevis* at the final time point and the fluorescence of *R. setigera* was enhanced in the presence of K. brevis at the 192-hour time point (Figure 4).

3.2 Inshore versus offshore

When the data from inshore species were combined, treating each species as a replicate, and compared to data from offshore data there was no significant difference between effects of *K. brevis* allelopathy on inshore vs. offshore competitors (Figure 2). Percent growth of inshore species was not significantly different than that of offshore species (t-test, n=4-5, p>0.99, Figure 2). Relative exponential growth rates of inshore competitors were also not significantly different from offshore species (t-test, n=4-5, p=0.66, Figure 2). The raw fluorescence of inshore and offshore species continued to increase in treatments exposed to *K. brevis* even if growth was inhibited (t-test, p=0.017,

p=0.021, p=0.018, n=5-6, Figure 3-4). *Karenia brevis* growth either increased or was maintained in all treatments.

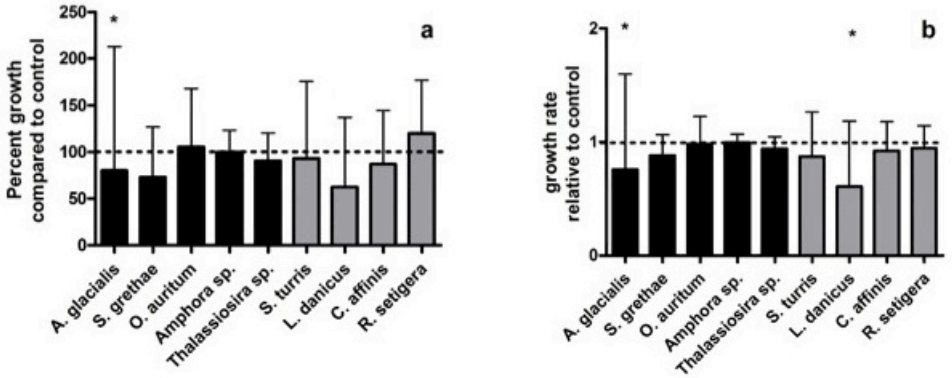

Figure 1. Effect of allelopathic *K. brevis* on competitor growth after 8-10 days exposure. Asterisks (*) indicate species whose growth was significantly affected by *K. brevis* in some way versus their own control. Black bars indicate species that predominantly occur inshore and gray bars are offshore species. Error bars represent ±1 SD. **a)** Percent growth relative to control of all species based on counts. The percent growth of *A. glacialis* was significantly inhibited (t-test, n=6, p=0.011). Dashed line indicates growth rate of control, 100. **b)** Growth rate relative to control of all species based on counts. The growth rate of *A. glacialis* and *L. danicus* were significantly inhibited by *K. brevis* (t-test, n=6, p=0.021, p=0.048 respectively). Dashed line indicates growth rate of control, 1.

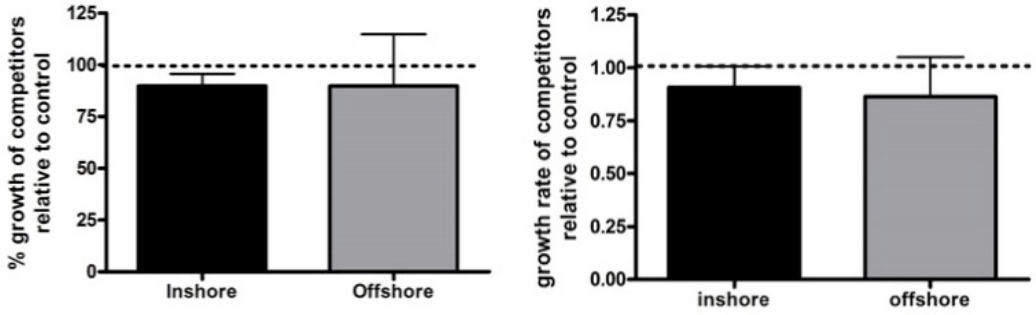

Figure 2. Effect of allelopathic *K. brevis* on growth of inshore competitors versus offshore competitors after 8-10 days exposure. Error bars represent ± 1 S.D. (**Left**) Average percent growth relative to control of all inshore competitors (n=5, black bar) versus all offshore competitors (n=4, gray bar) when exposed to *K. brevis*. Difference in mean % growth was not significant between inshore and offshore competitors (t-test,p=0.9969). (**Right**) Average growth rate relative to control of all inshore competitors (n=5, black bar) versus all offshore competitors (n=4, gray bar) when exposed to *K. brevis*. Difference in mean growth rate was not significant between inshore and offshore competitors (t-test, p=0.66).

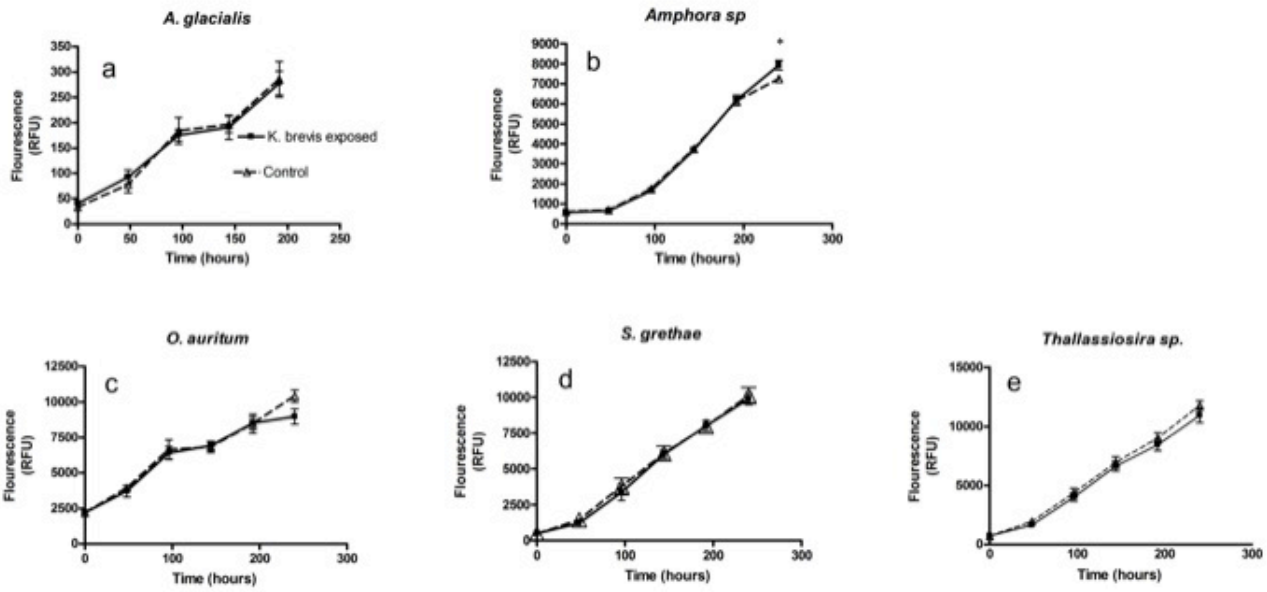

Figure 3. Fluorescence of inshore species measured every 48 for 192-240 hours. Error bars represent +/- 1 S.E.M. Rectangle points represent treatments expose to *K. brevis*, triangles represent those exposed to dilute media controls. (*) indicate a significant stimulation of fluorescence compared to control.

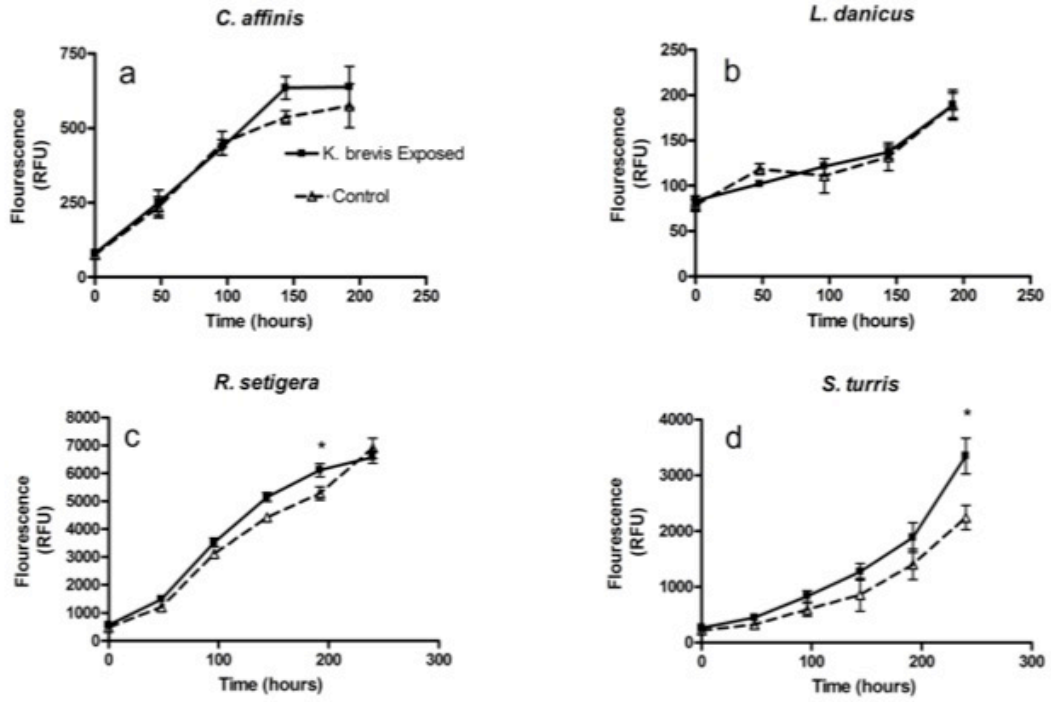

Figure 4. Fluorescence of offshore species measured every 48 for 192-240 hours. Error bars represent +/- 1 S.E.M. Rectangle points represent treatments expose to *K. brevis*, triangles represent those exposed to dilute media controls. (*) indicates a significant stimulation of fluorescence compared to control.

5. DISCUSSION

Inshore species and offshore species responded similarly to allelopathy from *K. brevis.* Only *A. glacialis* (inshore) and *L. danicus* (offshore) were significantly affected by *K. brevis* although several species trended toward being inhibited (Figure 1). *Thalassiosira* sp., *Amphora sp*., *O. auritum*, and *R. setigera* were found to resistant to *K. brevis* allelopathy (Figure 1). Thus, *K. brevis* was found to be allelopathic to a subset of phytoplankton competitors, but, there was no significant difference in the response of inshore species vs. offshore species (Figure 2). Among-species variation in response to allelopathy was similar to that seen in other studies and individual species responses were also mostly consistent with previous studies. As we observed in the current study, Poulson et al (2010) found that *A. glacialis* was significantly inhibited by *K. brevis*. In addition, Kubanek et al (2005) found that *O. aurita* was resistant to live *K. brevis* but *R. setigera* was susceptible to *K. brevis* allelopathy.

Competitor species habitat is not likely the underlying mechanism responsible for variation in species response to allelopathy, and species composition is not likely the reason *K. brevis* blooms occur where they do. Bloom initiation site could have been explained in part if offshore species had been more affected than inshore species. However, the data did not indicate a difference. The hypothesis was rejected because there was not a significant difference between effects on species in each environment (Figure 2).

Allelopathic compounds have been shown to reduce photosynthetic efficiency (Prince et al 2008). Both *R. setigera* and *S. turris* exhibited reduced growth in the presence of *K. brevis* but their fluorescence predicted that it was stimulated in the

presence of *K. brevis,* indicating damage to photosystem II (Figure 4). If photosystem II is damaged, energy can be released and picked up as fluorescence. Other species whose growth appeared to be marginally (but not significantly) diminished, also exhibited robust fluorescence, indicating sub-lethal damage to photosystem II.

In the current experiments, *K. brevis* and its competitors were separated by a mesh screen to ensure some other forms of competition, for example contact-based inhibition, were excluded. *Karenia brevis* could have used nutrients faster than the competitor but they could not pass through the filter to obtain nutrients. They were dependent on diffusion of nutrients through the mesh screen. A small degree of contamination by species crossing the filter was seen in this experiment. If extreme *K. brevis* contamination was seen in competitor cultures replicates were dropped. Contamination with competitor species was seen in few *K. brevis* cages. This should not have affected our conclusions since we were primarily interested in effects on competitors, not on *K. brevis*.

Other factors could explain species variation in response to allelopathy, such as growth stage and density of competitor, and can be explored in future experiments (Poulson et al 2010). Yamasaki et al (2011) found that population density of competitors, including *A. glacialis* and *Chaetoceros* spp., affected the response to *Skeletonema costatum* allelopathy. *Alexandrium fundyense* has also exhibited a species specific and density dependent allelopathic effect on its competitors (Hattenrath-Lehmann and Gobler 2011). More species could also be used to further explore this question.

In vitro results may also differ from results in the natural environment, since competitors normally come into direct contact with *K. brevis* and many other plankton

species. Poulson et al (2010) also found that allelopathic interactions with *K. brevis* could be overshadowed in ecologically complex environments. Mulderij et al (2007) found that allelopathy was also overshadowed in freshwater phytoplankton-macrophyte interactons.

Another mechanism is likely responsible for bloom location of *K. brevis*, such as environmental factors or differences in composition of other, non-phytoplankton organisms such as pathogens, grazers, and specifically, *Trichodesmium*, a cyanobacterium. Jonsson et al (2009) found that allelopathy is not responsible for bloom formation because allelochemicals do not reach high enough concentrations to be effective. The data supports this finding, indicating that the growth of offshore species is not significantly reduced by allelopathy from *K. brevis*. With both of these factors, it it not likely that allelopathy is responsible for the location of *K. brevis* bloom initiation sites.

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